REGULATORY T CELLS PROMOTE IL-17-DEPENDENT CARCINOGENIC IMMUNITY TO ENTEROTOXIGENIC *BACTEROIDES FRAGILIS*

by
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ABSTRACT

Many epithelial cancers are associated with chronic inflammation. However, the features of inflammation that are pro-carcinogenic are not fully understood. Regulatory T cells (Tregs) typically restrain overt inflammatory responses and maintain intestinal immune homeostasis. Their immune suppressive activity can inhibit inflammation-associated cancers. Paradoxically, we show that Tregs promote an oncogenic immune response to a common human symbiote associated with inflammatory bowel disease and colorectal cancer. Colonic Tregs initiate interleukin (IL)-17-mediated carcinogenesis in multiple intestinal neoplasia mice colonized with the human symbiote enterotoxigenic *Bacteroides fragilis* (ETBF). Depletion of Tregs in ETBF-colonized C57BL/6 Foxp3^{DTR} mice enhanced colitis but diminished tumorigenesis associated with shifting of the mucosal cytokine profile from IL-17 to interferon (IFN)-γ; inhibition of ETBF-induced colon tumorigenesis was dependent on reduced IL-17 inflammation but not dependent on IFN-γ. Treg enhancement of IL-17 production is cell-extrinsic. IL-2 blockade restored Th17 responses and tumor formation in Treg-depleted animals. Our findings demonstrate that Tregs limit the availability of IL-2 in the local microenvironment, suppressing excessive Th1-associated inflammation while permitting Th17 development necessary to promote ETBF-triggered neoplasia. Thus, we unveil a new mechanism whereby mucosal Treg responses to intestinal bacterial infection can promote tumorigenesis.

Table of Contents

Abstract ii

Table of Contents iii

List of Figures iv

Introduction 1

Materials and Methods 9

Results

Synchronized accumulation of activated mucosal Tregs and IL-17-producing cells precedes colon tumorigenesis in ETBF-colonized mice 13

Tregs promote colonic neoplasia in ETBF-colonized Min mice 15

Tregs provide help for Th17 responses to ETBF colonization via a cell-extrinsic mechanism 16

Tregs promote Th17 development in the colon via consumption of IL-2 19

Concluding Remarks 21

References 25

Figures 36

Curriculum vitae 55

List of Figures

Figure 1: ETBF-induced distal colon tumorigenesis 36

Figure 2: Accumulation of IL-17+ T cells in proximal and distal colons of ETBF-colonized mice. 37

Figure 3: Accumulation of Foxp3+ Tregs in proximal and distal colons of ETBF-colonized mice. 38

Figure 4: Accumulation of total CD4+ T cells in distal colons of ETBF-colonized mice. 39

Figure 5: Activated phenotype of CD4+ T cells and Tregs in colons of ETBF-colonized mice. 40

Figure 6: Treg depletion enhances inflammation but reduces microadenoma formation in Min mice. 41

Figure 7: ETBF colonization induces acute colitis at 1 week that is unaffected by Treg depletion. 42

Figure 8: Treg depletion mitigates the Th17 response to ETBF in favor of a Th1 response. 43

Figure 9: Mitigation of IL-17 response to ETBF in the absence of Tregs is not restricted to CD4+ (Th17) cells. 44

Figure 10: Mitigation of IL-17 response to ETBF in the absence of Tregs is independent of IFN-γ. 45

Figure 11: Reduced ETBF tumorigenesis in the absence of Tregs is independent of IFN-γ. 46

Figure 12: IL-17+ Foxp3+ Tregs infiltrate ETBF-infected colons. 47

Figure 13: Mixed chimeras reveal a cell extrinsic requirement for Foxp3 in Th17 differentiation. 48

Figure 14: Foxp3$^+$ Tregs are not necessary to maintain an established Th17 response. 50

Figure 15: Anti-IL-2 restores the Th17 response to ETBF in the absence of Foxp3$^+$ Tregs. 51

Figure 16: Anti-IL-2 restores the total tissue *Il17a* response to ETBF in the absence of Foxp3$^+$ Tregs. 52

Figure 17: Foxp3$^+$ Tregs do not inhibit Il2 transcription by effector T cells. 53

Figure 18: Anti-IL-2 restores ETBF-induced microadenoma formation yet minimizes colitis in the absence of Foxp3$^+$ Tregs. 54

Introduction

Humans are colonized with trillions of microbes, predominantly bacteria. Work by numerous investigators combined with the Human Microbiome Project (HMP), initiated in 2007, has provided remarkable data that has promoted our understanding of the complexity, variability and biology of human associations with our microbiota at diverse body sites[1]. Microbiota science has been fostered and complemented by microbial whole genome sequencing, technical sequencing advances, and rapidly evolving bioinformatics. The first phase of the HMP, completed in 2012, focused on establishing the 'normal' microbiota and now has evolved to address the key scientific challenge of translating what we learned to understand how the microbiota contributes to disease pathophysiology[2]. With this translation, there is optimism regarding the potential for development of new diagnostic and therapeutic approaches. This frontier has already yielded exciting observations in several fields such as atherosclerosis, obesity, and colon cancer[3-5]. This introduction will describe how *Bacteroides fragilis*, quite unexpectedly, became appreciated as having the capacity to act as either symbiote or pathogen, and it will describe gaps in our understanding of *B. fragilis* disease mechanisms, including that which is addressed in this dissertation.

All *B. fragilis* are obligate anaerobes that inhabit and flourish along the entire length of the colon where they are minority members of the normal colonic microbiota. Interest in *B. fragilis* blossomed in the 1970's with the recognition that these organisms were the leading anaerobes in bloodstream infection and critical contributors to intra-abdominal abscess formation[6,7]. Our subsequent understanding that *B. fragilis* is relatively aerotolerant (i.e., able to grow in the presence of nanomolar oxygen concentrations), at least in part, explains its success in mucosal colonization (where oxygen tension is higher), its survival following colon perforation when exposed to the peritoneal cavity prior to abscess formation, and its likelihood of inducing bacteremia[8]. The capsule of *B. fragilis* emerged as a pivotal virulence factor key to the organism's success in induction of abscess formation[9]. Molecular characterization of the first

polysaccharide purified from the surface of *B. fragilis*, polysaccharide A (PSA), revealed that PSA could both cause and induce protective immunity against abscess formation in animals[10,11]. Investigations of the capsule of *B. fragilis* and the host immune response to this organism led to two transformative observations. First, *B. fragilis* possess the most diverse surface polysaccharide gene repertoire of any known bacterium with the ability to synthesize up to 8 distinct capsular polysaccharides (A-H) that only populate the surface of *B. fragilis* one at a time[12]. The benefit to the organism of its ability to 'change its coat' remains unknown, for a single longitudinal human study suggests that *B. fragilis* polysaccharide expression is relatively stable in colonized human hosts[13]. Second, immunologic studies support PSA-expressing *B. fragilis* as a symbiote with the remarkable capacity to modulate homeostatic mucosal immunity as well as contribute to systemic immune development[14,15]. Most intriguingly, colonization with *B. fragilis* displaying PSA (strain NCTC9343) inhibits subsequent experimental chemically-induced (e.g., DSS, TNBS) colitis, likely mediated, in part, by enhanced bacterial:mucosal contact[16]. Hence, this strain is now proposed as a potential probiotic therapeutic to moderate inflammation in the colon. However, this concept is challenged by the fact that most individuals are already colonized by *B. fragilis*, and recent murine data suggests that once colonized with a *Bacteroides* species, mice resist re-colonization by a different strain of the same species[17].

Nearly in parallel with the early *B. fragilis* interest, in the 1980's, Myers and colleagues identified that select strains of *B. fragilis* accounted for a portion of diarrheal disease in lambs as well as other livestock including piglets, calves and foals[18-21]. They provided evidence that these strains of *B. fragilis* (ultimately named ETBF) induced intestinal secretion in association with disruption of the intestinal epithelial barrier and inflammation, in contrast to the symbiotic colonic associations of PSA-expressing *B. fragilis*. Importantly, this inflammatory experimental phenotype was dependent on a heat-labile factor secreted by the bacteria leading to the concept that a novel toxin-mediated colonic disease was induced by *B. fragilis*. As discussed in more detail below, the heat labile factor secreted by these colon disease-inducing strains of *B. fragilis*

was determined to be a metalloprotease protein toxin (most often termed *B. fragilis* toxin or BFT). Strains of *B. fragilis* possessing the toxin gene (*bft*) were termed enterotoxigenic *B. fragilis* (ETBF); whereas strains of *B. fragilis* lacking the *bft* gene were designated nonenterotoxigenic *B. fragilis* (NTBF).

Because enteric disease in humans is often a zoonosis, ETBF was subsequently sought as a cause of human diarrheal disease in a controlled study pioneered by Drs. Lyle Myers and R. Bradley Sack in 1992 in the pediatric outpatient clinics of the Apache Indian reservation in Whiteriver, AZ. This study revealed that children <1 year of age did not develop ETBF-associated diarrhea whereas diarrhea was associated with ETBF detection in children between the ages of one and five years. Importantly, *B. fragilis* was isolated from similar proportions of children in each age group[22]. Subsequently, the largest single study conducted in adults (older than 15 years, N=728) in Sweden identified ETBF in 27% of adults hospitalized with acute community-acquired diarrhea compared to 12% of 194 healthy outpatient controls. Although asymptomatic fecal detection of ETBF in the control groups of diarrheal disease studies from around the world was sometimes common, ranging from 0-29%, well-controlled epidemiological studies, nonetheless, repeatedly established an association of ETBF with human diarrheal disease[23].

A key clinical study conducted at the International Center for Diarrheal Disease Research (ICDDR,B) in Dhaka, Bangladesh changed the perspective on human ETBF disease. In this study, ETBF diarrheal disease was unexpectedly associated with fecal leukocytes and proinflammatory cytokines [interleukin (IL)-8 and tumor necrosis factor (TNF)-α], implicating ETBF as an etiology of inflammatory diarrhea disease[24]. Notably, the clinical observations of ETBF diarrhea at the ICDDR,B were consistent with the early observations in animals where colitis was observed in field and experimental infections. Consistent with the inflammatory potential of ETBF in the colon, limited studies to date suggest an association with active inflammatory bowel disease[25,26].

Altogether, these findings pointed towards acquisition of ETBF as a cause of acute, symptomatic inflammatory diarrhea and, further, that chronic asymptomatic colonization in humans of all ages was relatively common. However, our understanding of human ETBF epidemiology remains sparse. For example, we do not know whether asymptomatic colonization alone occurs in some hosts or stems from one of the frequent, but undiagnosed, diarrheal illnesses that all humans experience; whether human colonization begins most often in childhood and then persists; or whether some hosts can eliminate ETBF colonization through, as yet, unidentified immune mechanisms.

As mentioned previously, ETBF strains are so named because they secrete BFT, which remains the only known virulence factor for ETBF. Three BFT isotypes (encoded by the genes *bft-1, bft-2,* and *bft-3*) are known. BFT-2 exhibits greater specific activity as well as biologic activity in vitro and in vivo than BFT-1 with fewer details available regarding BFT-3[27-29]. To date, no ETBF strain has been identified with two different *bft* isotype genes and most contain one *bft* copy; occasional ETBF strains possess two *bft* gene copies of the same *bft* isotype.

Although also referred to as fragilysin in the literature, BFT does not exhibit cell lytic activity but rather promotes cell proliferation. The in vitro biologic activities identified for BFT occur swiftly and provide potential mechanisms by which ETBF induces inflammatory diarrhea and intersects with mechanisms of colon carcinogenesis. In colonic epithelial cell monolayers in vitro, BFT increases barrier permeability with active secretion of chloride ions, actions known to promote colon inflammation and carcinogenesis as well as diarrhea. BFT-induced increased barrier permeability correlates with cleavage of E-cadherin, an intercellular adhesion protein of the zonula occludens[30-32]. Although BFT is a protease toxin, the data do not support direct cleavage of E-cadherin by BFT, nor does BFT modify known protease-activated receptors (PARs) [33]. Rather, BFT acts via a specific, yet unknown, colonic epithelial cell surface receptor that precipitates E-cadherin cleavage. Processing of the intracellular E-cadherin fragment by host cell presenilin-1/γ-secretase follows initial shedding of the E-cadherin ectodomain.

Tethered to the intracellular domain of E-cadherin is β–catenin, a critical signaling molecule of the Wnt pathway whose actions are regulated, in part, by the adenomatous polyposis coli (APC) protein known to be mutated in the vast majority of human colorectal cancer (CRC) cases. Wnt signaling is one regulator of cell proliferation and, in the setting of mutant APC, enhanced β–catenin nuclear signaling results in dysregulated colonic epithelial cell proliferation. Consistent with this, Wu et al showed that BFT-induced degradation of E-cadherin augmented β–catenin nuclear signaling with induction of the oncogene *c-myc*, and upregulation of c-Myc expression contributed to BFT-induced colonic epithelial cell proliferation[34]. Other studies revealed alternative mechanisms by which BFT could be carcinogenic. Namely, BFT induces the polyamine catalyst, spermine oxidase (SMO) that triggers reactive oxygen species (ROS) production, DNA damage and cell proliferation[35].

The range of colonic epithelial cell signal transduction activated by BFT is remarkably broad and incompletely understood. For example, BFT also activates nuclear factor (NF)-κB and mitogen-activated protein kinase signaling in colonic epithelial cells resulting in their release of pro-inflammatory chemokines (such as IL-8, TNF-α among others) [36,37]. These chemokines are released across the basolateral membrane of colonic epithelial cell monolayers where they can foster recruitment of neutrophils and other immune cells to the colonic mucosa. BFT also induces colonic epithelial cell expression of cyclooxygenase (COX)-2 (but not COX-1), increasing mucosal prostaglandin E_2[38]. These mechanisms likely contribute to ETBF-induced inflammatory diarrhea in animals and humans.

Thus, cleavage of E-cadherin, activation of NF-κB (with anti-apoptotic effects), increased polyamine metabolism and induction of DNA damage within the colonic epithelium are key BFT oncogenic mechanisms identified to date in in vitro studies. E-cadherin cleavage yields multiple pro-carcinogenic outcomes that are well demonstrated in murine models of colon carcinogenesis, including Wnt signaling, colonic epithelial cell proliferation[39] and epithelial barrier disruption that promotes mucosal inflammation and colon tumor formation[40,41]. Additionally, colonization

with ETBF in patients with CRC might contribute to cancer metastasis because epithelial tumors with reduced E-cadherin exhibit increased metastatic potential. Pro-oncogenic activities of NF-κB and SMO activation include induction of mucosal inflammation[57-59 60], enhanced epithelial cell survival or proliferation, DNA damage, and/or promotion of angiogenesis[42,43].

The clinical observation that ETBF causes human inflammatory diarrhea combined with the in vitro studies of BFT mechanism(s) of action led to the hypothesis that ETBF were carcinogenic bacteria. This hypothesis was tested in mice chronically colonized with ETBF (*bft2*). Notably, germ-free mice developed lethal colitis in 24 hrs when colonized with ETBF but not NTBF; whereas a single ETBF inoculation of conventional C57Bl/6 mice resulted in acute, occasionally bloody, inflammatory diarrhea that gradually subsided over a week in nearly all mice[44]. Despite resolution of symptoms, colonization persisted, lasting up to one year, and was resistant to treatment with anti-anaerobic antibiotics. Chronic ETBF colonization induces persistent, low level, IL-17A-dominant colon inflammation with modest hyperplasia, foci of Stat3 activation, reactive oxygen species production, and DNA damage[45]. ETBF strains with an in-frame chromosomal deletion of *bft* do not induce colitis, and conversely, transfection of NTBF with a plasmid bearing *bft* induces colitis similar to wild-type ETBF, demonstrating the central contribution of BFT ('necessary and sufficient') to ETBF disease[44].

The carcinogenic potential of ETBF was demonstrated in multiple intestinal neoplasia (Min) mice. Min mice and humans with familial adenomatous polyposis (FAP) are genotypically similar, possessing a heterozygous nonsense mutation in the tumor suppressor gene *Apc*. However, Min mice and FAP patients exhibit phenotypically distinct disease. Whereas the majority of FAP patients develop many colorectal adenomatous polyps, adenoma formation is largely restricted to the small bowel of Min mice[46]. However, when colonized with ETBF, Min mice developed inflammatory colitis that progressed to gross distal colon tumorigenesis, resembling the vast majority of human CRC cases, within four weeks[47]. This distal colon localization of ETBF-induced tumors in Min mice occurs in spite of relatively uniform ETBF

mucosal colonization along the colonic axis. Time course studies revealed that ETBF colonization of Min mice rapidly initiated tumorigenesis, with excess histologic microadenomas identified within 1-2 weeks after ETBF colonization, and lethal tumor burdens resulting in animal death within ~3 months. In contrast, histologic microadenomas are rare in sham or NTBF-colonized mice at early time points, and they survive at least 5 months.

Mechanistically, ETBF induces widespread, immediate (within 2 days) then prolonged, activation of signal transducer and activator of transcription-3 (Stat3) in colonic epithelial cells and associated infiltrating immune cells. In the microenvironment of ETBF colon tumors, Stat3 activation is accentuated compared to parental Min mouse colon tumors where more modest Stat3 activation is detected. Predictably, since activation of the Stat3 transcription factor is integral to adaptive $CD4^+$ T helper type 17 (Th17) cell differentiation, IL-17A-secreting Th17 cells dominate the early ETBF-associated mucosal inflammatory immune response. IL-17 blockade as well as depletion of CD4 cells inhibits ETBF colon tumorigenesis confirming that ETBF triggers Th17-dependent carcinogenesis[47]. IL-17 is a potent chemoattractant for neutrophils, and neutrophils are prominent in ETBF colitis, likely releasing mutagenic ROS[48]. ETBF also induces SMO expression in vivo, and treatment of Min mice with an SMO inhibitor reduced ETBF-induced colonic inflammation, epithelial cell proliferation and colon tumorigenesis[35].

Thus, these ETBF murine models confirmed the in vitro activities of BFT to induce NF-κB, SMO and Wnt signaling, ROS production and DNA damage. Most importantly, the oncogenic potential of ETBF was confirmed, and for the first time, adaptive immune responses, specifically Th17 adaptive immunity, were identified as carcinogenic. Since these observations, Th17 immunity has been identified to contribute to carcinogenesis in numerous models and in human disease. Activation of the Stat3/IL-17 pathway, in fact, correlates with a poorer prognosis in human CRC[49].

In an attempt to further characterize the regulation of pro-carcinogenic IL-17 responses induced by ETBF colonization, we studied the role of $Foxp3^+$ regulatory T cells (Tregs). FOXP3

is a critical nuclear transcription factor that imparts immunosuppressive functions to most Tregs. In fact, defects in *Foxp3* result in the development of immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, or devastating autoimmune diseases[50]. Tregs play a necessary immunosuppressive role that protects against immune-mediated pathologies. Therefore, we expected that Tregs would diminish the magnitude of pro-carcinogenic ETBF-induced IL-17 responses and thus mitigate tumor formation. This notion is supported by data indicating that depletion of Tregs ultimately leads to the development of colitis in mice not challenged with any colitogenic microbes, and adoptive transfer of Tregs with naïve effector ($Foxp3^-$) $CD4^+$ T cells prevents the development of experimental colitis in conventional mice[51-53]. Furthermore, Treg specific deletion of Stat3 results in the ultimate development of spontaneous Th17 colitis[52].

We found that ETBF colonization was characterized by the accumulation of activated Treg cells, as well as $IL\text{-}17^+$ T cells, at the dominant site of ETBF tumorigenesis, the distal colon. Surprisingly, we observed that depletion of Tregs in ETBF-colonized Min mice led to the abrogation of tumorigenesis at the earliest stages. Colons of Treg-depleted ETBF-colonized animals were highly inflamed, demonstrating that the suppressive capacity of colonic Tregs remained intact. Notably, the inflammation in Treg-depleted ETBF-colonized animals that exhibited increased colitis, but decreased tumorigenesis, was characterized by elevated IFN-γ and profoundly decreased IL-17A in the lamina propria, resulting in loss of the characteristic Th17 colitis normally associated with ETBF colonization. We show that Tregs promote acute IL-17-driven colitis via local consumption of IL-2, which inhibits Th17 polarization while enhancing expansion of Th1 cells. While mucosal Tregs initially promote Th17 polarization, they do not participate in the stabilization of the IL-17 response at later stages of ETBF colitis. Thus, we identify an unexpected role for Tregs in promoting the early stages of immune carcinogenesis.

Materials and Methods

Mice and reagents

C57BL/6 Foxp3$^{DTR-GFP}$ mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, New York). C57BL/6 Rag1$^{-/-}$, CD45.1, and IFN-γ$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Apc*$^{\Delta 716}$ (Min) mice were obtained from Drs. David Huso and Bert Vogelstein. IFN-γ$^{-/-}$ x Foxp3$^{DTR-GFP}$ mice were obtained in our facility by crossing C57BL/6 Foxp3$^{DTR-GFP}$ mice with IFN-γ$^{-/-}$ mice. Min x Foxp3$^{DTR-GFP}$ mice were obtained in our facility by crossing C57BL/6 Foxp3$^{DTR-GFP}$ mice with Min mice. In some experiments, bone marrow (BM) chimera mice were established by retro-orbital injection of 10^7 BM cells from donor mice into sub-lethally irradiated (300 rad) recipient C57BL/6 Rag1$^{-/-}$ mice. Irradiated mice were rested 6 hours before BM cell injection. Reconstituted mice were maintained on prophylactic antibiotic treatment (Sulfatrim) prior to ETBF inoculation (see below). In accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care International, mice were maintained under specific pathogen-free conditions and studied according to protocols approved by the Johns Hopkins University Animal Care and Use Committee.

Aqua cell viability dye was purchased from Life Technologies (Grand Island, NY). Flow cytometry antibodies against CD3, CD4, CD45.2, Foxp3, IL-17A, and IFN-γ were purchased from Affymetrix/eBioscience (San Diego, CA). Intracellular staining (ICS) was performed with eBioscience Foxp3 staining kit according to the manufacturer's instructions. Anti-IL-2 mAb (clone S4B6) and isotype control (rat IgG2a) were purified from hybridomas purchased at American Type Culture Collection (Manassas, VA).

ETBF mouse models

ETBF strain 86-5443-2-2 was used in this study [44,47]. *B. fragilis* strains were grown anaerobically on BHI medium plates containing 37 g of brain heart infusion base (Difco Laboratories, Detroit, MI) per liter along with 5 g of yeast extract (Difco) per liter, 0.1 mg of vitamin K per liter, 0.5 mg of hemin per liter, 50 mg of L-cysteine, and 6 μg of clindamycin per liter (all from Sigma, St. Louis, MO). A single colony was inoculated into BHI broth and grown anaerobically overnight at 37°C. Pelleted, washed bacteria were resuspended in 0.1 N sodium bicarbonate buffer and adjusted to an optical density corresponding to ~10^9 colony forming units (CFU)/ml for mouse inoculations. Three to 4 week old mice were treated with clindamycin and streptomycin (0.1 g/L and 5 g/L, respectively, in water bottles) 5 days prior to ETBF inoculation by gavage (~10^8 bacteria in PBS). BM chimera mice were inoculated with ETBF 6 weeks after sub-lethal irradiation. All strains are resistant to these antibiotic treatments. Mice were sacrificed 7 days after colonization, unless otherwise noted.

Treg depletion and IL-2 neutralization

Mice received 50 ng intraperitoneal (ip) injections of diphtheria toxin (DT; Sigma-Aldrich, MO) per g body weight on days one and two prior to ETBF inoculation (D-2, D-1) and on days 1, 3 and 5 after ETBF inoculation (D+1, D+3, D+5). Colons were harvested from mice 7 days after ETBF inoculation. In some experiments, IL-2 was neutralized by ip injection of 0.5 mg/mouse of anti-IL-2 mAb (clone S4B6) or isotype control (rat IgG2a) 2 days prior and every day after ETBF inoculation until mouse sacrifice. For tumor experiments, mice received 50 ng DT per g body weight on D-1, D0, D+1, D+3, and every other day until sacrifice and harvest on D+13.

Lamina propria lymphocytes (LPL) isolation

Dissected colons were flushed with 20 ml Ca^{2+}, Mg^{2+} free PBS 1X and cut longitudinally. Tissues were cut in <0.5 mm pieces and washed 3 times for 20 min in 37ºC 2mM EDTA, 10% FCS, 25mM Hepes, HBSS buffer. Tissue pieces were subsequently digested 30 min in 5% FCS RPMI

in presence of 400 Unit/ml Liberase (Roche Diagnostic, Indianapolis, IN) and 0.2 mg/ml DNAse 1 (Roche Diagnostic). Mononuclear cells were isolated by 20/40/80 Percoll gradient separation (GE Healthcare Life Science, Pittsburgh, PA).

Flow cytometry and fluorescence-associated cell sorting (FACS)

Colons from 1-2 mice per group were processed to obtain lamina propria lymphocytes (LPLs) as previously described [54]. Mononuclear cells collected by Percoll gradient separation were cultured 4hr in Iscove's Modified Dulbecco's Medium (IMDM) with 5% FCS and in the presence of Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience). Cells were then washed and stained for cell surface markers followed by fixation and permeabilization (Foxp3 Fixation buffer, eBioscience). ICS was performed for IFN-γ, IL-17A and Foxp3. Flow cytometry acquisition was performed on LSRII cytometer (BD Bioscience) and data was analyzed using FACSDiva 6.1.3 software. In some experiments, FACS was performed on FACSAria II (BD Bioscience).

Quantitative RT-PCR

Total RNA was isolated from sorted cells or whole tissue using *TRIzol*® reagent from Life Technologies (Grand Island, NY) according to manufacturer's instructions. 1μg of RNA was reverse transcribed using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems®). 40 cycles of TaqMan® Gene Expression qRT-PCR was performed on 1 ng RNA per sample for indicated genes. ^Ct was calculated by subtracting Ct of *Gapdh* from Ct of target gene and averaging 2 technical replicates.

Histology and microadenoma counts

Colons were dissected and preserved in 10% buffered formalin. Histologic examination was performed after hematoxylin and eosin (H&E) staining of 5 μm sections. To facilitate

longitudinal examination of the full-length colon, colons were 'Swiss-rolled' prior to embedding and sectioning. Total colon inflammation was scored as previously described[44]. Microadenoma counts on formalin-fixed paraffin-embedded H&E colon tissue sections from 2-week ETBF-colonized Min mice, and all histopathology scoring, were performed by a pathologist (DH).

Statistical analysis

Comparison of means was done by unpaired, two-tailed Mann-Whitney U testing, unless otherwise indicated. A p value of ≤ 0.05 was considered to designate a significant difference.

Results

Synchronized accumulation of activated mucosal Tregs and IL-17-producing cells precedes colon tumorigenesis in ETBF-colonized mice

ETBF colonization of four to five week old C57Bl/6 mice ($Apc^{+/+}$) elicits acute, self-limited (~3-4 days) inflammatory diarrhea, associated with a robust mucosal IL-17 response. ETBF colonization then persists, leading to a chronic (≥1 year), asymptomatic Th17-mediated colitis[44,45]that promotes distal colon tumorigenesis in Min mice[47] (**Figure 1**). Since IL-17A is required for ETBF-triggered tumorigenesis, we sought to understand the regulation of inflammation and colon tumorigenesis in the context of ETBF colonization. Because mucosal Tregs are instrumental in tuning the intestinal Th17 response to counter microbial aggression[55] or, conversely, to limit excessive responses to microbial components[51,56], we looked for a possible Treg response to ETBF. We found that early after ETBF colonization (day 7) IL-17^+ and $Foxp3^+$ T cell density (cells per gram of tissue) and percent of lymphocytes concurrently increased in the tumor-prone distal colon compared to sham (uninfected) mice (**Figure 2 and 3**). While there was little difference in the density and percentage of IL-17^+ T cells between the proximal and distal colon of ETBF-colonized mice, the density, but not percentage, of Tregs that accumulated in the distal colon was significantly greater than that in the proximal colon of ETBF-colonized mice **(Figure 2 and 3)**. This suggests that the density of total $CD4^+$ T cells may be increased in the distal colon of ETBF-colonized mice, which is indeed the case **(Figure 4)**. In addition, the proportion of $CD4^+$ and $Foxp3^+$ T cells with an activated phenotype, characterized by increased expression of CD44 and CD69 (**Figure 5A)**, was dramatically increased in the distal, but not the proximal, colon after 7 days colonization with ETBF compared to sham. Tregs in ETBF-colonized mice also expressed KLRG1 **(Figure 5B)**, which putatively indicates increased suppressive function, at least in vitro[57]. This was interesting because the ligand for KLRG1 is E-cadherin; however, in vivo experiments to address the functional role of $KLRG1^+$ Tregs, with or without ETBF, were unsuccessful with variable results (data not shown). Nonetheless, the

synchronized expansion of activated and IL-17-producing T cells as well as activated Treg cells following ETBF colonization suggests that Treg responses may play a role in modulating IL-17-driven immune pro-carcinogenesis in the colon.

Tregs promote colonic neoplasia in ETBF-colonized Min mice

We previously showed that ETBF-induced colon tumorigenesis is IL-17-dependent since injection of an anti-IL17 blocking antibody inhibited tumor formation[47]. Foxp3$^+$ Treg function in intestinal mucosa is typically thought to suppress excessive effector immune responses to microbiota and protect the integrity of the intestinal barrier[58-60]. Thus, we initially hypothesized that Tregs are necessary to limit ETBF-induced IL-17-mediated tumorigenesis. To assess the impact of Tregs on ETBF tumorigenesis, we crossed Min mice to Foxp3$^{DTR\text{-}GFP}$ mice that express the diphtheria toxin receptor (DTR) on Foxp3$^+$ cells[61]. Intraperitoneal administration of DT, therefore, selectively depletes Foxp3$^+$ cells. Because autoimmunity develops when Tregs are systemically depleted for more than two weeks[61], we tested the impact of Treg depletion on early microadenoma induction by ETBF in Min mice, which develop microadenomas in the distal colon beginning one week after ETBF colonization. This early time window (day 13) allowed us to assess the effect of Foxp3$^+$ cells on early ETBF tumorigenesis prior to any systemic effects of Treg depletion. DT or PBS was administered to Min x Foxp3^{DTR} mice starting one day prior to ETBF inoculation and every other day thereafter until harvest at day 13. Not surprisingly, Treg-depletion significantly increased colonic inflammation (**Figure 6A and 6B**), emphasizing that the immunosuppressive function of colonic Tregs remains intact. Surprisingly, however, microadenoma formation was significantly reduced in Treg-depleted mice compared to Treg sufficient controls (**Figure 6A and 6B**).

During the acute colitis stage at one week, inflammation upon Treg depletion was similar to that of Treg-competent mice in sham (0.0 +/- 0.0, mean +/- SD in sham and sham + DT mice, $p>0.999$, N=7-8 mice per group) or ETBF-colonized mice (1.78 +/- 0.67 vs 2.1 +/- 0.88, ETBF vs ETBF + DT mice, $p=0.5$, N=9-10 mice per group)(**Figure 7A and 7B**). Of note, one week ETBF-colonized mice with or without Treg depletion displayed marked colitis compared to sham mice ($p<0.0001$ for both comparisons, **Figure 7A and 7B**).

Tregs provide help for Th17 responses to ETBF colonization via a cell-extrinsic mechanism

Since IL-17 induction is an absolute requirement for ETBF-induced colon tumorigenesis [47] and microadenoma formation was significantly reduced in ETBF-colonized Treg-depleted mice, we asked whether Tregs in ETBF-colonized mice modified the mucosal Th1/Th17 balance in favor of protumoral Th17 effectors, potentially contributing to the development of distal colon tumorigenesis. To determine the impact of Tregs on the Th1/Th17 balance in ETBF-colonized mice, lamina propria lymphocytes (LPLs) were isolated after one week from the colons of sham or ETBF-colonized $Foxp3^{DTR\text{-}GFP}$ mice treated or not with DT and analyzed for cytokine production by intracellular staining (ICS). Indeed, in accordance with the unexpected finding that Treg depletion diminished early in situ tumor formation, depletion of Tregs in ETBF-induced colitis dramatically reduced the proportion of Th17 cells (5 +/- 2%, N=5) in the colonic lamina propria compared to ETBF-colonized Treg-sufficient mice (27 +/- 8%, N=5, $P < 0.0001$ **Figure 8A and 8B**). Notably, the effect of Treg depletion was not limited to Th17 cells as IL-17 production by other cell types ($CD4^-$) was also mitigated (**Figure 9**). Conversely, $Foxp3^+$ Treg depletion in ETBF-colonized mice strongly enhanced the Th1 response with a marked increase in the proportion of IFN-γ^+ $CD4^+$ T cells. Since IFN-γ^+ $CD4^+$ T cells also increased in sham mice after Treg depletion, our data further support the concept that mucosal regulation of Th1 by Tregs is key to intestinal immune homeostasis (**Figure 8A**).

Since STAT1/IFN-γ signaling is well established as an inhibitor of the STAT3/Th17 pathway [62], we explored the possibility that loss of IL-17 production upon Treg depletion was indirectly due to inhibition by the increased Th1 response. We crossed $Foxp3^{DTR}$ mice to IFN-$\gamma^{-/-}$ mice and demonstrated that, even though T cell-generated IL-17 did trend slightly higher in IFN-$\gamma^{-/-}$ mice compared to IFN-$\gamma^{+/+}$ mice after Treg depletion, in the absence of IFN-γ, Treg depletion still mitigated the proportion of Th17 cells in LPLs (**Figure 8B and 10**). This result demonstrated that decreased IL-17 production in Treg-depleted mice is mostly *not* due to increases in IFN-γ. Because Treg cells are viewed as components of the tumor microenvironment (TME) that

suppress anti-tumor immunity and promote tumor growth [63], it is possible that Treg depletion impaired ETBF tumorigenesis by unleashing a robust anti-tumoral IFN-γ response. Thus, we asked whether increased IFN-γ driven inflammation in the absence of Tregs might promote potent anti-tumor immunity. Depletion of Tregs in Min x $Foxp3^{DTR}$ x IFN-$\gamma^{-/-}$ mice reduced microadenoma numbers similar to those observed in Treg-depleted Min x $Foxp3^{DTR}$ mice, establishing that decreased Treg-mediated IL-17 production, and not increased IFN-γ, is most likely responsible for reduced neoplasia (**Figure 11**).

While these results suggest that Tregs are providing cell-extrinsic "help" for the differentiation of naïve $CD4^+$ LPL to Th17, it is possible that the effects of $Foxp3^+$ cell depletion could be cell-intrinsic, i.e. via depletion of $Foxp3^+$ precursors to the colonic Th17 cells. Indeed, there is evidence that Th17 cells and peripherally induced Tregs may differentiate from a common $Foxp3^+ROR\gamma t^+$ precursor, or Th17 cells may result from "trans-differentiation" of Tregs [64,65]. Consistent with a potential intrinsic mechanism of Foxp3 in cells differentiating into Th17 cells, we indeed observed the presence of IL-17^+ $Foxp3^+$ $CD4^+$ T cells in colon tumors (**Figure 12)**.

To distinguish whether Foxp3 marked precursors to Th17 cells (intrinsic mechanism) or if $Foxp3^+$ Treg help was necessary for Th17 differentiation (extrinsic mechanism) in ETBF colitis, we transferred both $CD45.2^+$ $Foxp3^{DTR}$ and $CD45.1^+$ $Foxp3^{WT}$ bone marrow (BM) at a 1:1 ratio into sub-lethally irradiated $RAG1^{-/-}$ recipients, establishing mixed BM chimera mice. This approach allowed us to selectively deplete $CD45.2^+$ $Foxp3^{DTR}$ Tregs following DT injection, and it enabled us to monitor the origin ($CD45.1^+$ or $CD45.2^+$ BM) of the Th17 cells during ETBF colitis (**Figure 13A**). Upon $CD45.2^+$ $Foxp3^+$ depletion, a cell intrinsic mechanism would only diminish Th17 responses among $CD45.2^+$ T cells while a cell extrinsic mechanism would not diminish Th17 responses among either $CD45.1^+$ or $CD45.2^+$ T cells. When $CD45.2^+$ $Foxp3^{DTR}$ cells were depleted in the mixed BM chimeras, we found that neither $CD45.1^+$ (i.e. $CD45.2^-$) nor $CD45.2^+$ Th17 cells were decreased compared to Treg sufficient mixed BM chimeras (**Figure**

13B). In these experiments, CD45.2^{+} Tregs were fully depleted, but CD45.1^{+} Tregs were unaffected (**Figure 13B**). As a positive control for the action of DT, we depleted total Tregs from ETBF-colonized CD45.2^{+} Foxp3^{DTR} BM chimeras. As expected, this resulted in a Th17 proportion in LPLs similar to the sham mixed BM chimera mice (**Figure 13B**). In order to determine at which point Tregs enhanced ETBF-induced Th17 responses, DT was administered one day post ETBF inoculation (late depletion), in contrast to the experiments described above in which DT was given two days prior to ETBF inoculation. In striking contrast to Treg depletion prior to ETBF colonization, late depletion of Tregs resulted in a Th17 response that was comparable to that of Treg sufficient ETBF-colonized mice (**Figure 14**). Taken together, these results suggest that Foxp3^{+} Tregs provided extrinsic support for initial Th17 differentiation, but not maintenance, in response to ETBF. Regardless of when Treg depletion was initiated (early versus late), the absence of Tregs resulted in an increased proportion of Th1 cells (**Figure 14**).

Tregs promote Th17 development in the colon via consumption of IL-2

Tregs, which express high levels of IL-2 receptors, particularly when activated, do not produce endogenous IL-2. Thus, they are extremely dependent on exogenous sources of IL-2 for their survival. Treg proliferation and activation upon ETBF colonization may therefore deprive the local inflammatory environment of IL-2. Since IL-2 down-regulates IL-17 production via STAT5 signaling, Tregs may promote Th17 differentiation by limiting the amount of local IL-2 available to uncommitted T cells [66-68]. In contrast, IL-2 may promote proliferation of Th1 cells [69]. To determine whether Treg promotion of Th17 differentiation upon ETBF colonization occurs via limiting local IL-2, ETBF-colonized Treg-depleted Foxp3^{DTR} mice were injected with a blocking antibody against IL-2 (S4B6-1) and LPLs were isolated after 7 days of ETBF colonization. **Figure 15** shows that anti-IL-2 treatment indeed restored the Th17 response to ETBF in the absence of Treg cells. In contrast, when Treg cells were present, anti-IL-2 treatment minimally increased the proportion of Th17 cells (**Figure 15A and 15B**). In keeping with the notion that IL-2 expands Th1 cells, anti-IL-2 treatment partially mitigated the increase in IFN-γ-producing cells upon Treg depletion (**Figure 15A**). When Tregs were depleted, the expression of total IL-17 mRNA was similarly restored when anti-IL-2 mAb was administered (**Figure 16**). The decreased availability of IL-2 likely resulted from cytokine consumption by Tregs ("sink effect") and not inhibition of IL-2 production by effector cells as IL-2 mRNA expression was indeed similar between effector T cells (CD11b- Foxp3- CD4+ or CD8+) sorted from LPLs of ETBF-colonized Treg-sufficient and Treg-deficient mice (**Figure 17**). As expected, expression of Foxp3 was low in both groups of effector T cells, and IL-17 mRNA was decreased in effector T cells sorted from Treg-depleted mice compared to effector T cells from Treg-sufficient mice (**Figure 17**).

Importantly, when IL-17 is increased in Treg-depleted Min x Foxp3^{DTR} mice treated with anti-mouse IL-2 mAb, microadenoma formation was restored (**Figure 18A**), demonstrating that the IL-17-dependent protumoral activity of mucosal Tregs in the ETBF-driven TME requires IL-2 deprivation. Despite the restoration of microadenomas upon anti-IL-2 treatment of Foxp3-

depleted mice, colonic inflammation was reduced compared to Treg-depleted isotype-treated Min x Foxp3^{DTR} mice (**Figure 18B**). The enhanced colitis of Treg depletion was also reduced in Treg-depleted IFN-$\gamma^{-/-}$ mice (**Figure 18B**). These results dissociate colitis from tumorigenesis in that Th1-mediated colitis upon Treg depletion (**Figure 8A**) does not induce microadenomas as Th17-mediated colitis does (**Figure 6B**). Thus, we conclude that Treg cells facilitate Th17 differentiation in the inflammatory microenvironment of the ETBF-colonized colon by limiting excess IL-2 that can otherwise prevent the development of a pro-carcinogenic Th17-mediated immune response.

Concluding remarks

In an effort to further understand the mechanisms of ETBF-driven procarcinogenic colitis, we investigated the impact of mucosal Treg cells on the inflammatory response to ETBF murine colonization. These studies led to the surprising conclusion that Tregs are critical to initiate the Th17 colitis necessary for tumor induction. Through consumption of IL-2, Tregs inhibit the development of Th1 colitis, which is not procarcinogenic, and shift T cell differentiation to Th17 in the lamina propria of ETBF-colonized mice. This helper role of Tregs for Th17 development occurs only at the very initial stages of ETBF-mediated colitis.

Recent reports showed that mucosal Tregs, which commonly orchestrate mucosal immune homeostasis by restraining inflammatory responses to microbiota in the gut, can cooperate with immune effector cells to protect against and eradicate infections[55,67]. Th17 cells, by their production of IL-17, are predicted to be important to the mucosal defense against ETBF, resulting in the recruitment of bactericidal polymorphonuclear and phagocytic mononuclear cells. Consistent with this idea, IL-17$^{-/-}$ mice colonized with ETBF exhibit increased morbidity and mortality compared to wild-type mice (C Dejea, C Sears, unpublished). However, the mucosal Th17 defenses against ETBF are not antiseptic and thus do not prevent chronic colitis associated with the persistence of both ETBF and mucosal IL-17 production[45]. In conjunction with a host genetic predisposition, such as the *Apc* mutation in Min mice, ETBF-induced mucosal IL-17 is instrumental in promoting marked distal colon tumorigenesis[47]. Importantly, a predominant IL-17 response is associated with worse survival in human CRC[49]. ETBF represents the first common human commensal identified as a potent trigger for murine colon tumorigenesis[47,70]. Surprisingly, ETBF triggers its carcinogenic IL-17 response, in part, through the accumulation of mucosal Tregs. This is in stark contrast to the mucosal immune homeostasis proposed as resulting from nontoxigenic *B. fragilis*-mediated Treg induction[14,16]. Our results show that ETBF-mediated colitis drives the accumulation of Foxp3^{+} Tregs that, despite their suppression of Th1 mucosal inflammation, consume IL-2 resulting in Th17 polarization that is critical to ETBF tumorigenesis.

IL-2 is a potent inhibitor of IL-17 production via competition between STAT5 (IL-2 signaling) and STAT3 (IL-17 signaling) for binding to the *Il17* promoter [71]. Further, Tregs are incapable of producing IL-2 and are highly dependent on exogenous sources of IL-2, indispensable to their survival[72,73]. Thus, Tregs capture IL-2 in their environment via their elevated constitutive expression of the high affinity IL-2 receptor, CD25. By modulating levels of exogenous IL-2, Tregs release the STAT5 inhibitory effect on Th17 differentiation of uncommitted $CD4^+$ T cells or suppress already-committed effector T cells by IL-2 deprivation[73,74]. Although Tregs have also been shown to inhibit IL-2 production by effector T cells (endogenous IL-2) [73,75], we have shown herein that ETBF-induced Tregs do not alter IL-2 transcription by lamina propria effector T cells. Importantly, our results establish that Tregs strictly intervened at the initiation stage of Th17 differentiation, but they are not required for the stabilization of the colonic Th17 immune signature since late Treg depletion does not impair the Th17 response to ETBF infection **(Figure 14)**. Our findings described here may be related to those of Pandiyan *et al*. who showed in a murine model of oral *Candida albicans* that poorly suppressive Tregs were important to initiate the anti-fungal Th17 response, but they eventually resumed their suppressive function and inhibited pathogenic Th17 effectors [67].

Because Treg and Th17 cells demonstrate functional plasticity, we experimentally ruled out the possibility that ablation of Foxp3 limited Th17 cells generated via elimination of $Foxp3^+$ progenitors. Tregs have been reported to convert into IL-17 or IFN-γ-producing cells [76-78], and Th17 cells have been reported to up-regulate Tbet and produce IFN-γ[79,80] or up-regulate Foxp3 and acquire suppressive function[65,81]. Xu *et al* demonstrated that, in the presence of IL-6, $Foxp3^+$ Tregs can be induced to become Th17 cells. Moreover, IL-17^+RORγt^+ Tregs have been detected in mouse and human cancer and have been shown to be highly pathogenic[82]. Even though a small proportion of IL-17^+Foxp3^+ T cells was detected in the colons of ETBF-colonized mice (**Figure 12**), our use of mixed BM chimeras demonstrated that ablation of $CD45.2^+$ BM derived $Foxp3^+$ Tregs did not impair the generation of $CD45.2^+$ Th17 cells when $CD45.1^+$ Tregs were present.

This indicated Tregs act to promote Th17 differentiation via an extrinsic mechanism in the ETBF murine model.

Our study showed that, in the absence of Tregs, ETBF colonization induced a strong inflammatory response associated with increased IFN-γ production. Although commonly associated with anti-tumor host defenses, we demonstrated that the IFN-γ/Th1-type immune response was not responsible for reduced colon neoplasia in Treg depleted-ETBF-colonized Min mice. This result highlights the impact of IL-17 in tumor initiation. The increased IFN-γ response to Treg depletion is not surprising, especially in the gut where there is constant exposure to inflammatory stimuli such as pathogen-associated molecular patterns on commensal bacteria. In fact, Treg cell depletion has recently been shown to increase the inflammatory cytokines IFN-γ and IL-17 in the intestines [51], and adoptive transfer of Treg cells can cure experimental models of inflammatory bowel disease[53,83]. These data emphasize that Tregs exercise strong immunosuppressive function on effector T cells in other settings. However, in ETBF colonization, mucosal Tregs polarize a robust IL-17 mucosal immunity while suppressing Th1 immunity. We noted that the inflammation score one week following ETBF colonization, in contrast to inflammation following 13 days of ETBF colonization, remained similar with or without Tregs; however, the character of this inflammation differed, with Tregs promoting IL-17 while repressing IFN-γ.

Our findings allow a better understanding of the Th17 polarization induced by ETBF colonization and its contribution to colon tumorigenesis. However, the cellular and molecular mechanisms by which ETBF selectively increases Tregs in the distal murine colon remain unknown. As BFT, which profoundly alters the biology of colonic epithelial cells[84], is required for ETBF-driven oncogenic IL-17 production, we postulate that epithelial-derived signals, following the rapid cleavage of E-cadherin induced by BFT[32], contribute to the recruitment of effector immune cells, including Tregs. It has recently been shown that diminished epithelial barrier function induced the production of alarmins that directly recruit and activate Tregs[85]. For

example, IL-33 stimulated Tregs and opposed the restraining effect of proinflammatory IL-23 on Tregs in microbiota-induced chronic colitis. Additional inflammatory signals, including those potentially provided by BFT-altered epithelial cells, may play a critical role in the Treg response to ETBF and promotion of protumoral IL-17 production. Furthermore, while the precise mechanism by which IL-17 contributes to ETBF-mediated tumorigenesis is still unclear, IL-17 has been shown to directly influence colon epithelial cell signaling, survival, and proliferation [86]. The elucidation of IL-17-induced epithelial-derived signaling pathways will contribute to a better understanding of the regional distribution of ETBF-induced colon tumors and may eventually provide therapeutic targets to control deleterious mucosal IL-17 and Treg responses.

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Figures

Figure 1: ETBF-induced distal colon tumorigenesis. Methylene blue-stained colon of 2 month ETBF-colonized Min mouse. The distal colon predominance of ETBF tumorigenesis is shown (white arrows).

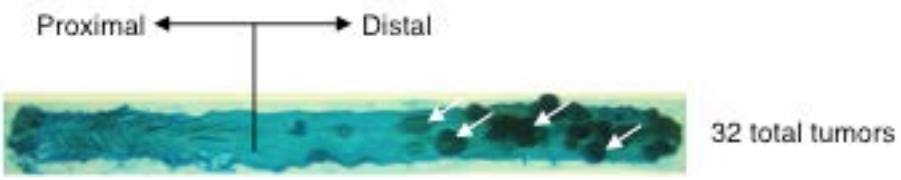


Figure 2: Accumulation of IL-17+ T cells in proximal and distal colons of ETBF-colonized mice. Lamina propria lymphocytes (LPLs) were isolated from proximal and distal colons (as demarcated in **Figure 1**) of C57Bl/6 mice 7 days after sham (PBS) or ETBF inoculation and 4 hr culture with Cell Stimulation Cocktail preceded intracellular staining (ICS) for IL-17A. Live $CD3^+$ $IL\text{-}17^+$ cell numbers were normalized by the mass of colon tissue after cleaning and before LPL isolation (top). Percent $IL\text{-}17^+$ of live $CD3^+$ lymphocytes (bottom). Each symbol represents one sample, and error bars represent one standard deviation from the mean in each direction. Shown are data from two separate experiments. Pc = proximal colon, Dc = distal colon

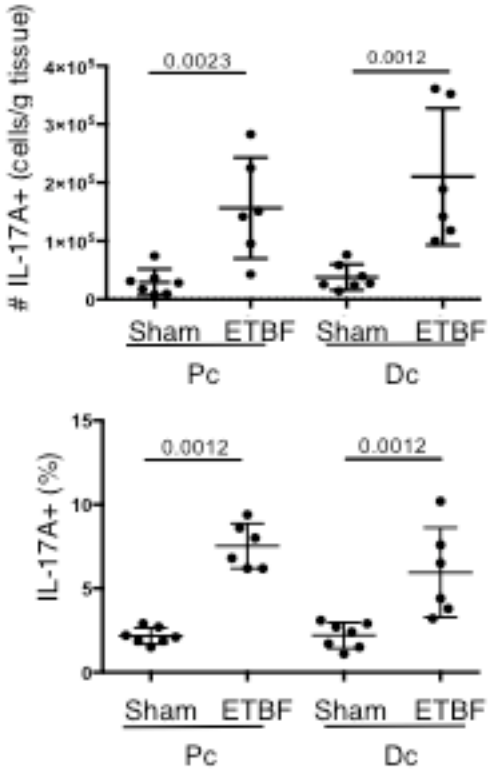


Figure 3: Accumulation of Foxp3+ Tregs in proximal and distal colons of ETBF-colonized mice. LPLs were isolated from proximal and distal colons of C57Bl/6 mice 7 days after sham (PBS) or ETBF inoculation. ICS for Foxp3 on proximal and distal colon LPLs from C57Bl/6 mice was performed fresh ex vivo without stimulation. Live $CD3^+$ $CD4^+$ $Foxp3^+$ cell numbers normalized by mass of colon tissue as in **Figure 2** (top). Percent $Foxp3^+$ of live $CD3^+$ $CD4^+$ lymphocytes (bottom). Each symbol represents one sample, and error bars represent one standard deviation from the mean in each direction. Shown are data from two separate experiments. Pc = proximal colon, Dc = distal colon

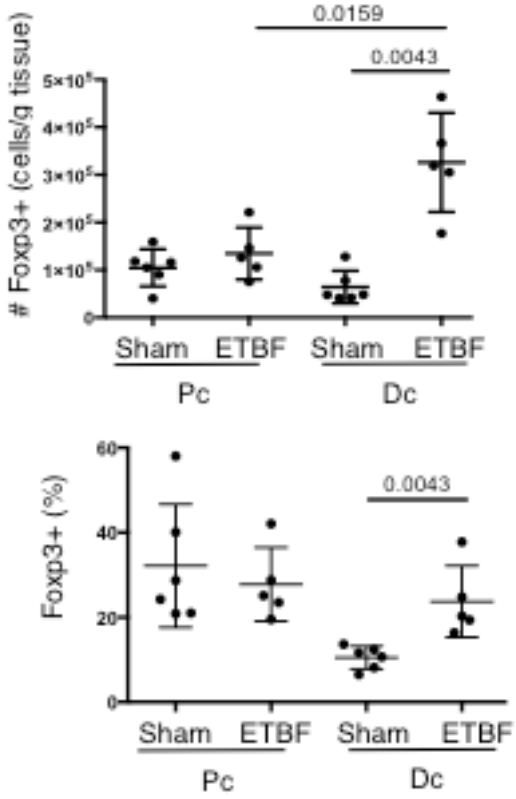


Figure 4: Accumulation of total CD4+ T cells in distal colons of ETBF-colonized mice. Live $CD3^+$ $CD4^+$ LPL numbers from **Figure 3** normalized by mass of colon tissue. Each symbol represents one sample, and error bars represent one standard deviation from the mean in each direction. Shown are data from two separate experiments. Pc = proximal colon, Dc = distal colon

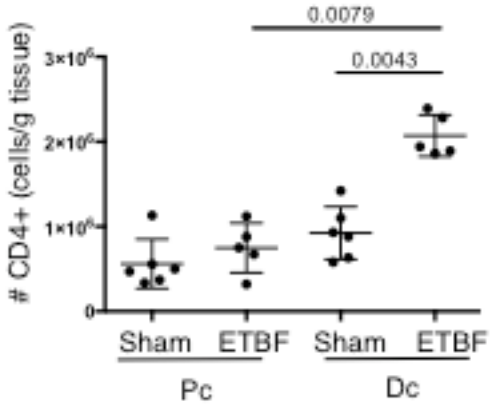


Figure 5: Activated phenotype of CD4+ T cells and Tregs in colons of ETBF-colonized mice. LPLs were isolated from proximal and distal colons of C57Bl/6 mice 7 days after sham (PBS) or ETBF inoculation. Surface staining and ICS for Foxp3 on proximal and distal colon LPLs from C57Bl/6 mice was performed fresh ex vivo without stimulation. A) Percentages are of live $CD3^+$ $CD4^+$ lymphocytes (top) and of live $CD3^+$ $CD4^+$ $Foxp3^+$ lymphocytes (bottom) in the indicated quadrant. B) Histogram of live $CD4^+$ $Foxp3^+$ cells; percentages of $KLRG1^+$ Tregs from distal colon of ETBF-colonized mice (58.3%) and sham mice (27.3%). Results shown are representative of two independent experiments. Pc = proximal colon, Dc = distal colon

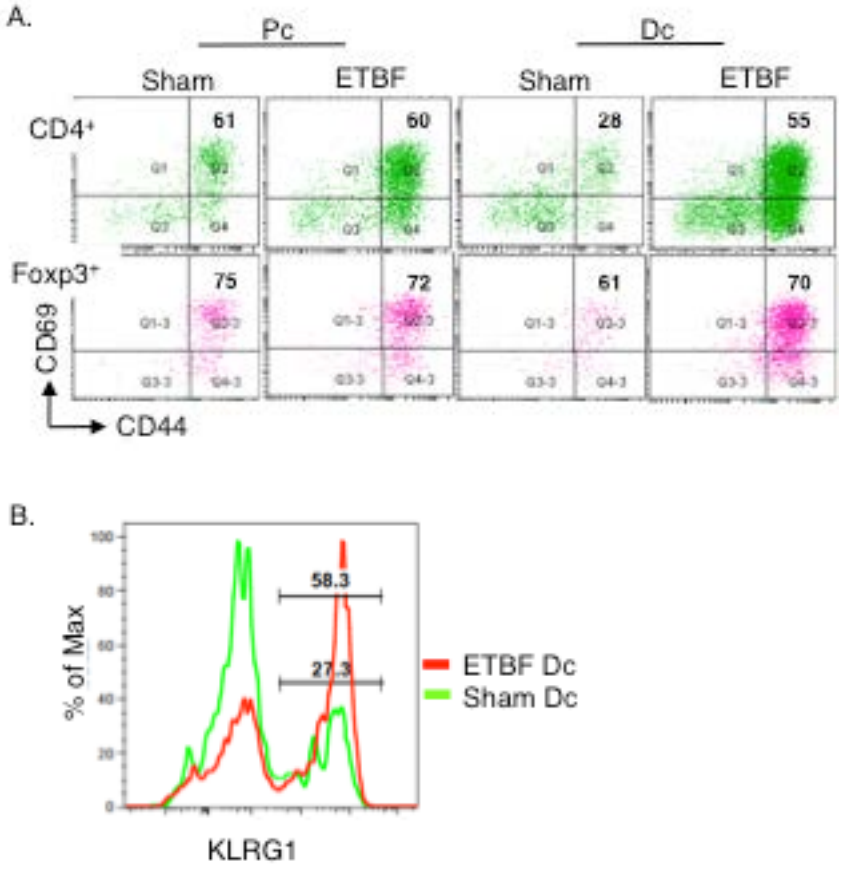


Figure 6: Treg depletion enhances inflammation but reduces microadenoma formation in Min mice. B6.Foxp3^{DTR}xMin mice were inoculated with ETBF on day 0. Either 150 µl purified sterile H_2O or 50 ng/g diphtheria toxin (DT) was administered intraperitoneal (ip) on days -1, 0, 1, 3, and every other day until sacrifice and harvest. Colons were harvested on day 13, cleaned, rolled, and fixed in 10% formalin for histology & scoring. A) Images are of distal colon. Microadenoma is encircled. Scale bars are 50 µm. B) Each symbol represents total inflammation score (left) or microadenomas counted (right) per Min mouse colon from **Figure 6A.** Data shown include 2 separate experiments with 5-12 mice per group per experiment. Bars indicate mean +/- SD.

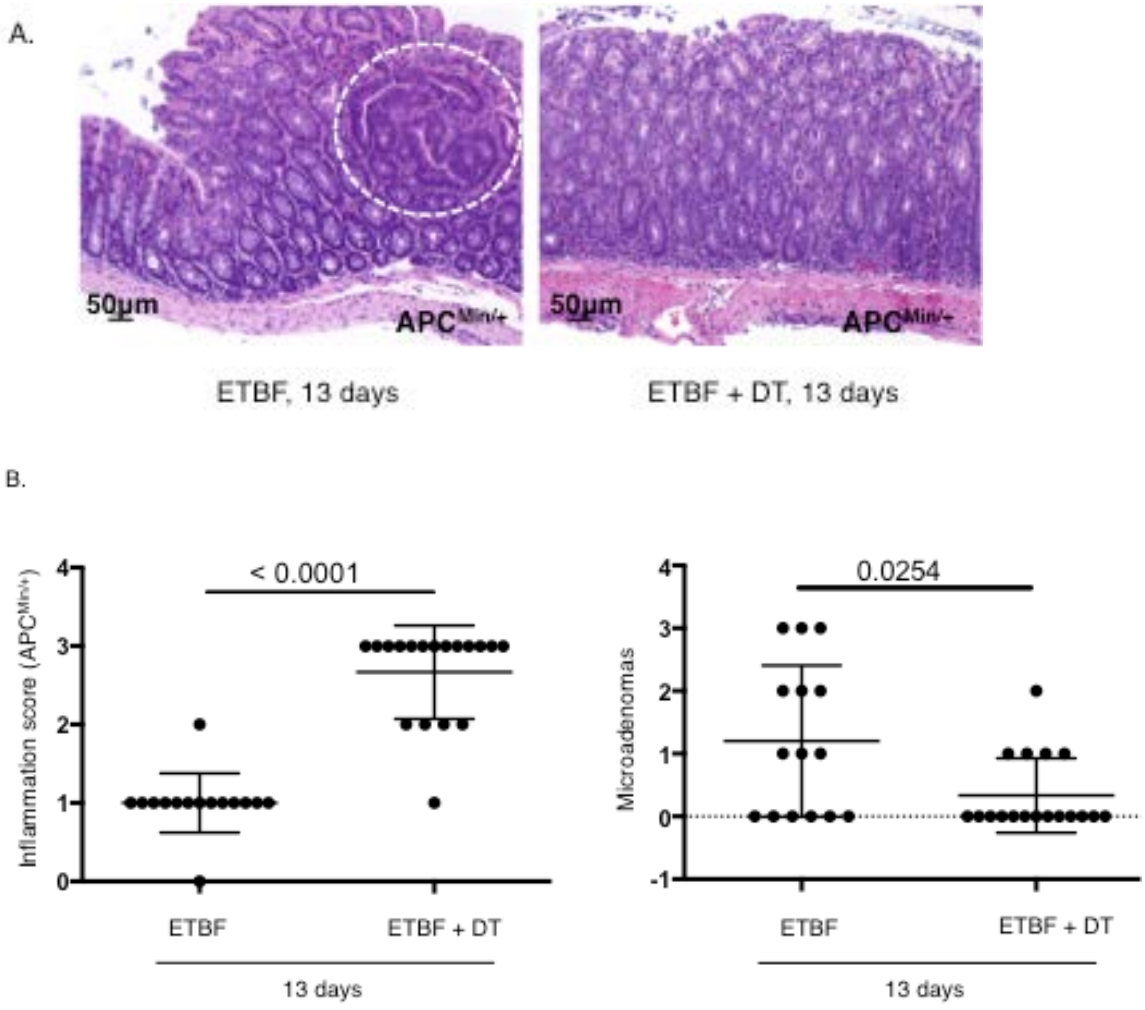


Figure 7: ETBF colonization induces acute colitis at 1 week that is unaffected by Treg depletion. B6.Foxp3^{DTR} mice were inoculated with sham or ETBF on day 0. Either 150 µl purified sterile H_2O or 50 ng/g diphtheria toxin (DT) was administered intraperitoneal (ip) on days -2, -1, 0, 1, 3, and 5 until sacrifice and harvest. Colons were harvested on day 7, cleaned, rolled, fixed in 10% formalin, paraffin embedded, and H&E stained for histology & scoring. A) Images are of distal colon. Scale bars are 50 µm. B) Each symbol represents total inflammation score per mouse colon from **Figure 7A**. Data shown include 2 separate experiments with 2-6 mice per group per experiment. Bars indicate mean +/- SD.

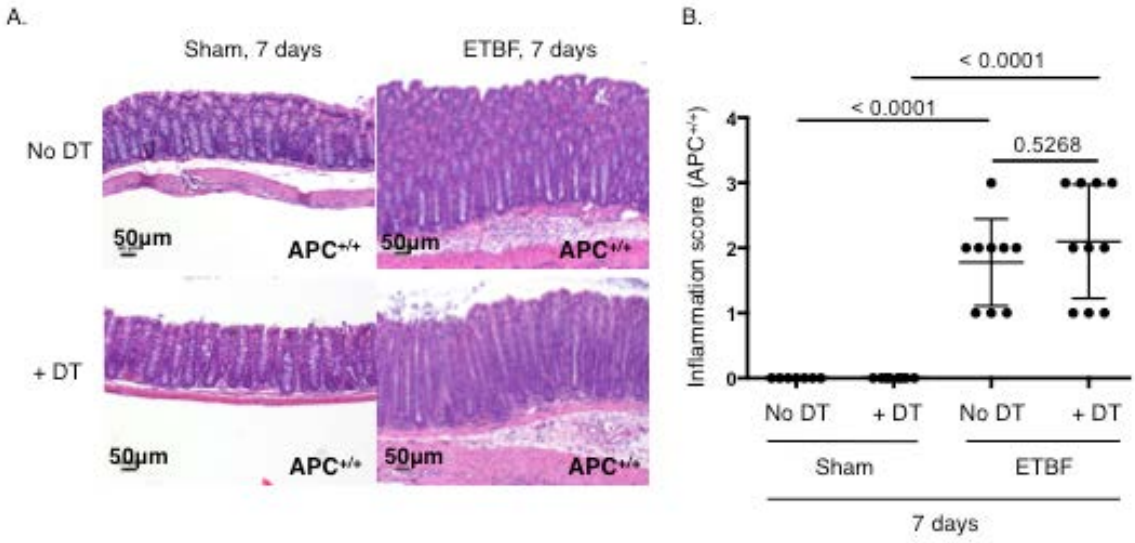


Figure 8: Treg depletion mitigates the Th17 response to ETBF in favor of a Th1 response. Colon LPLs from 1-2 B6.Foxp3DTR mice per group were harvested on day 7 following inoculation with ETBF or Sham on day 0. Either 150 µl purified sterile H2O or 50ng/g DT was administered ip on days -2, -1, 1, 3, & 5. Cells were stimulated ex vivo, followed by ICS. A) Plots show viable CD3+ CD4+ Foxp3- LPLs and are a representation of 4-5 separate experiments. B) Aggregate data from combined experiments showing percentage of viable CD3+ CD4+ Foxp3- LPLs that are IL-17A+. Each symbol represents 1-2 Treg-depleted B6.Foxp3DTR mice (●, +DT) or Treg-sufficient mice (○, No DT), and error bars represent 1 standard deviation from the mean in each direction. Holm-Sidak method for multiple comparisons was used to compare No DT vs + DT groups.

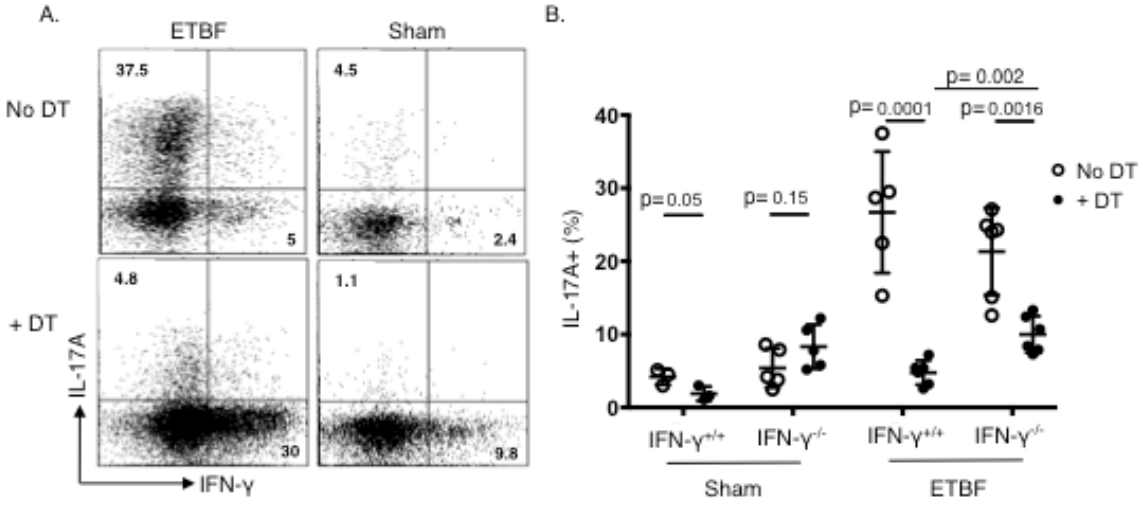


Figure 9: Mitigation of IL-17 response to ETBF in the absence of Tregs is not restricted to CD4+ (Th17) cells. B6.Foxp3DTR mice were inoculated with ETBF. Either 150 µl purified sterile H2O or 50 ng/g DT was administered ip on days -2, -1, 1, 3, & 5. Colonic LPLs from each mouse were harvested on day 7. Cells were stimulated ex vivo, followed by ICS. Dot plots show viable LPLs from 1 mouse per group and are representative of 2 experiments.

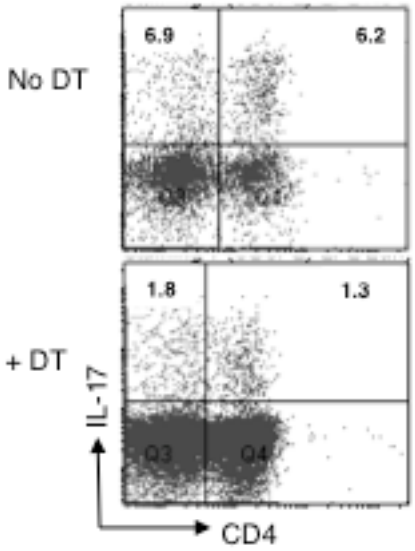


Figure 10: Mitigation of IL-17 response to ETBF in the absence of Tregs is independent of IFN-γ. B6.Foxp3^{DTR} and B6.Foxp3^{DTR}xIFNγ$^{-/-}$ mice were inoculated with ETBF on day 0. Either 150ul purified sterile H_2O or 50 ng/g DT was administered ip on days -2, -1, 1, 3, & 5. Colonic LPLs from 1-2 mice per group were harvested on day 7. Cells were stimulated ex vivo, followed by ICS. Dot plots show viable CD3$^+$ CD4$^+$ Foxp3- LPLs and are representative of 4-5 experiments.

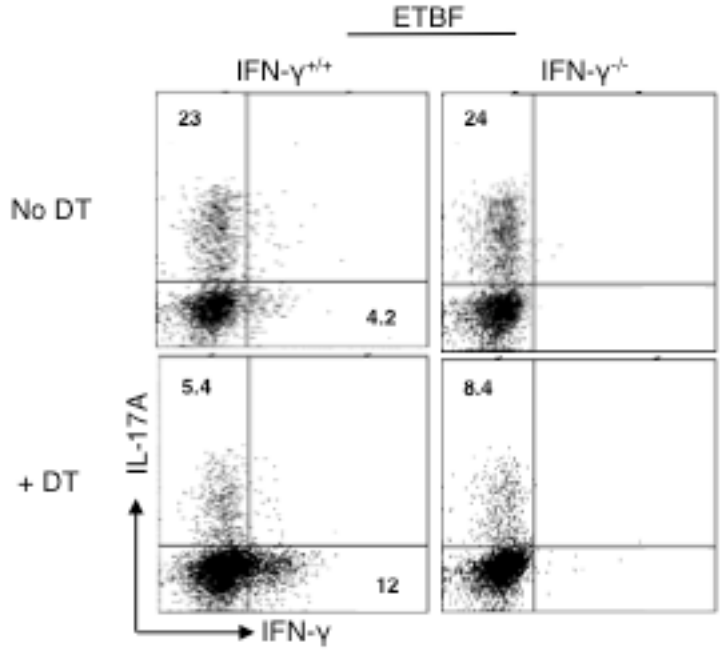


Figure 11: Reduced ETBF tumorigenesis in the absence of Tregs is independent of IFN-γ. B6.Foxp3^{DTR} x IFNγ$^{-/-}$ x Min mice were inoculated with ETBF on day 0. Either 150 µl purified sterile H_2O or 50 ng/g DT was administered ip on days -1, 0, 1, 3, and every other day until sacrifice and harvest. Colons were harvested on day 13, cleaned, rolled, and fixed in 10% formalin, paraffin embedded and H&E stained for microadenoma counting per colon. Each symbol represents 1 colon, and error bars represent 1 standard deviation from the mean in each direction. 2-5 mice per group per experiment. Combined data from 2 experiments. IFNγ$^{+/+}$ x Min microadenoma counts from **Figure 6B** are also shown for easier side-by-side comparison.

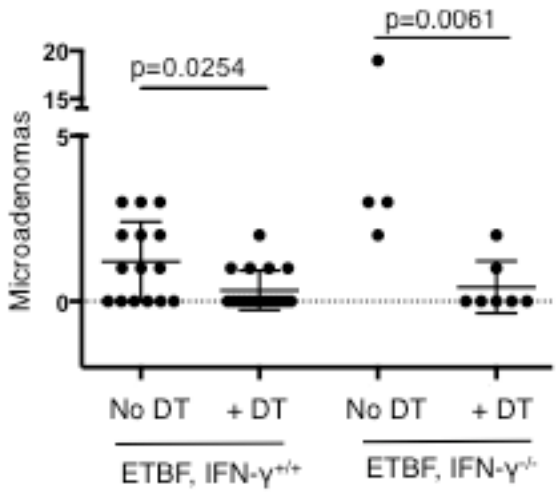


Figure 12: IL-17+ Foxp3+ Tregs infiltrate ETBF-infected colons. Min mice were inoculated with ETBF on day 0. At 10 weeks, colonic LPLs were harvested from colon tumors (left) and adjacent non-tumor colon tissue (right). Cells were stimulated ex vivo, followed by ICS. Dot plots show viable CD4+ LPLs. Percentage of IL-17+ within Foxp3+ gate is indicated.

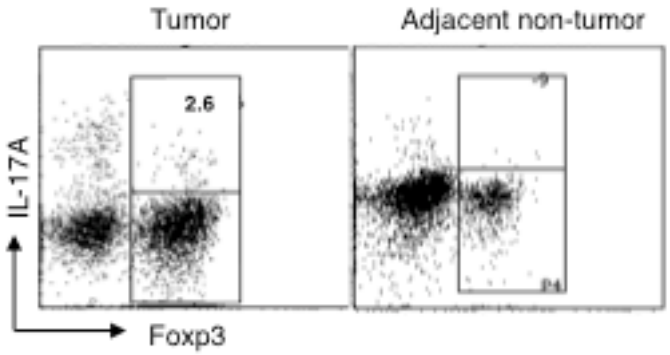
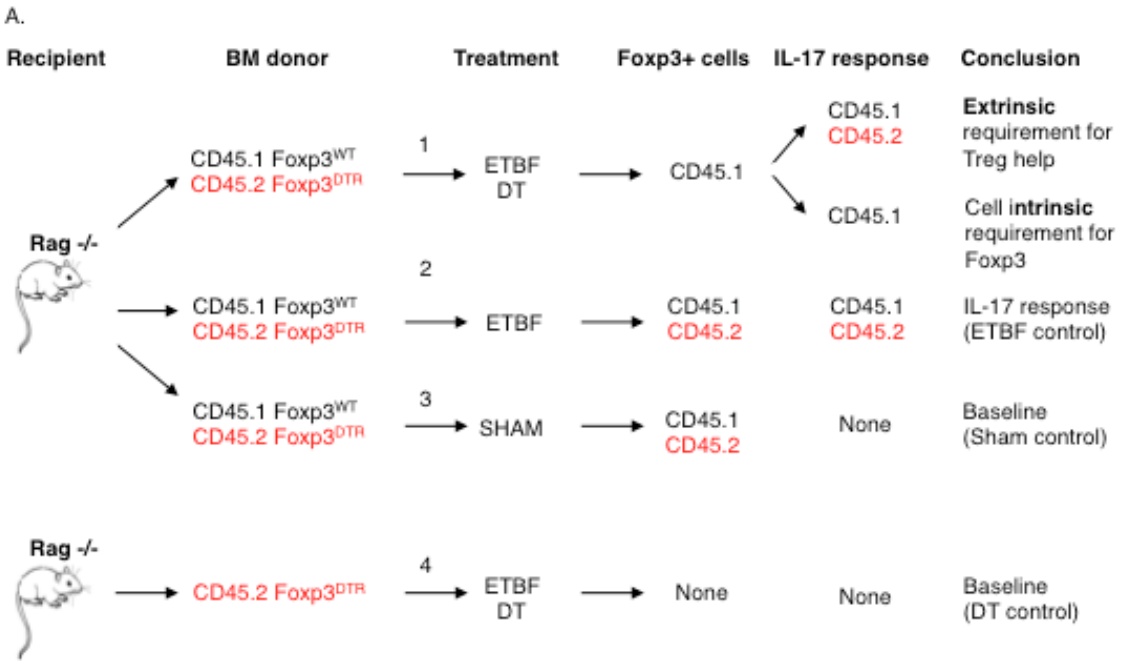


Figure 13: Mixed chimeras reveal a cell extrinsic requirement for Foxp3 in Th17 differentiation. A) Schematic of the experiment designed to determine whether Foxp3 expression is a cell extrinsic or intrinsic requirement for Th17 differentiation. B) 10^7 total bone marrow cells from B6.Foxp3^{DTR} CD45.2 mice only (DT control), or mixed 1:1 with bone marrow cells from B6.Foxp3^{WT} CD45.1 mice, were transferred retro-orbitally into Rag1$^{-/-}$ recipients that had received 300 rads irradiation 5-6 hours prior. Following 6 weeks for hematopoietic cell reconstitution, mice were inoculated with ETBF or sham on day 0, and either 150 µl purified sterile H_2O or 50 ng/g DT was administered ip on days -2, -1, 1, 3, & 5. Colonic LPLs from 1-2 mice were harvested on day 7 following ETBF or Sham inoculation. Cells were stimulated ex vivo followed by ICS. Dot plots show viable CD3$^+$ CD4$^+$ LPLs and are representative of 2 experiments.



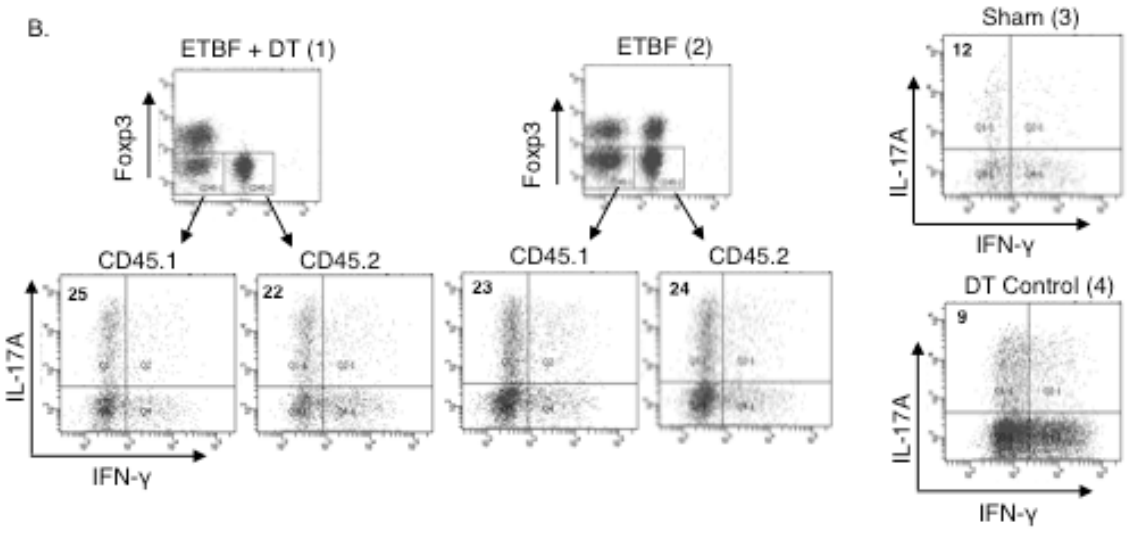

B.
ETBF + DT (1)
Foxp3
CD45.1
CD45.2
25
22
IL-17A
IFN-γ
ETBF (2)
Foxp3
CD45.1
CD45.2
23
24
Sham (3)
12
IL-17A
IFN-γ
DT Control (4)
9
IL-17A
IFN-γ

Figure 14: Foxp3$^+$ Tregs are not necessary to maintain an established Th17 response. Colon LPLs from 1 B6.Foxp3^{DTR} mouse per group were harvested on day 7 following inoculation with ETBF or sham on day 0. For no depletion and early depletion, purified sterile H_2O or DT, respectively, was administered ip on days -2, -1, 1, 3, & 5. For late depletion, DT was administered ip on days 1, 3, & 5. Cells were stimulated ex vivo, followed by ICS. Plots show viable CD3$^+$ CD4$^+$ Foxp3- LPLs and are representative of 2 mice per group.

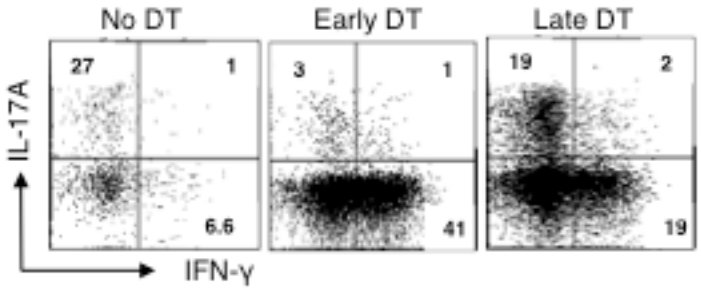


Figure 15: Anti-IL-2 restores the Th17 response to ETBF in the absence of Foxp3$^+$ Tregs. 4-9 B6.Foxp3DTR mice per group were inoculated with ETBF. Either 150 µl purified sterile H_2O or 50 ng/g DT was administered ip on days -2, -1, 1, 3, & 5, and either rat anti-mouse IL-2 (S4B6-1) or rat IgG2a isotype (JES3-19F) was delivered ip daily (day -2 through day 6). Colonic LPLs from each mouse were harvested on day 7. Cells were stimulated ex vivo, followed by ICS. A) Representative dot plots show viable CD3$^+$ CD4$^+$ Foxp3- LPLs from 1 mouse per group. aIL-2, anti-mouse IL-2. B) Aggregate data from **Figure 15A** showing percentage of viable CD3$^+$ CD4$^+$ Foxp3- LPLs that are IL-17A$^+$. Each symbol represents one B6.Foxp3^{DTR} mouse, and error bars represent 1 standard deviation from the mean in each direction. Isotype-treated animals were not different from untreated animals, so these two animal groups were combined.

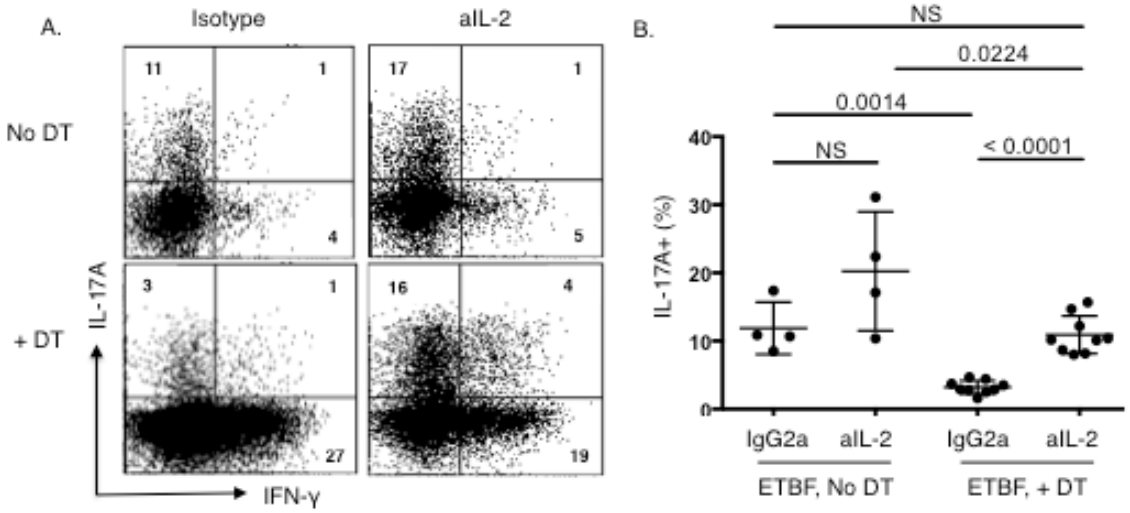


Figure 16: Anti-IL-2 restores the total tissue *Il17a* response to ETBF in the absence of Foxp3^{+} Tregs. Tissue for RNA isolation was taken from the middle colon from each mouse in **Figure 15B** and Taqman qRT-PCR was performed for IL-17A. ^Ct was calculated by subtracting Ct of *Gapdh* from Ct of IL-17A and averaging 2 technical replicates. Each symbol represents one mouse, and error bars represent 1 standard deviation from the mean in each direction.

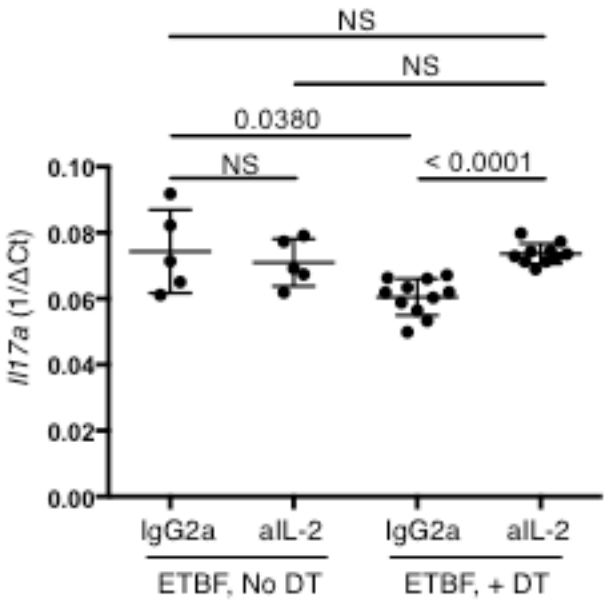


Figure 17: Foxp3^{+} Tregs do not inhibit Il2 transcription by effector T cells. 6-12 B6.Foxp3^{DTR} mice per group were inoculated with ETBF on day 0. Either 150 µl purified sterile H_2O or 50 ng/g DT was administered ip on days -2, -1, 1, 3, & 5. Colonic LPLs from 1-3 mice per group were harvested on day 7 for fluorescence-associated cell sorting. Effector T cells (CD11b-, Foxp3-, CD4+ or CD8+) were sorted from Treg-depleted mice (●, +DT) and from Treg-sufficient mice (O, No DT). RNA was isolated from Trizol and Taqman qRT-PCR was performed for indicated genes. *Gapdh* was used as housekeeping control for total RNA quantity (^Ct), and average of 2 technical replicates were used for each sample. CD4+ Foxp3+ Tregs were sorted from Treg-sufficient mice as the reference sample for calculating ^^Ct. Fold change = 2^-(^^Ct). Each symbol represents one mouse (or pooled group), and error bars represent 1 standard deviation from the mean in each direction. Holm-Sidak method for multiple comparisons was used to compare No DT vs + DT groups.

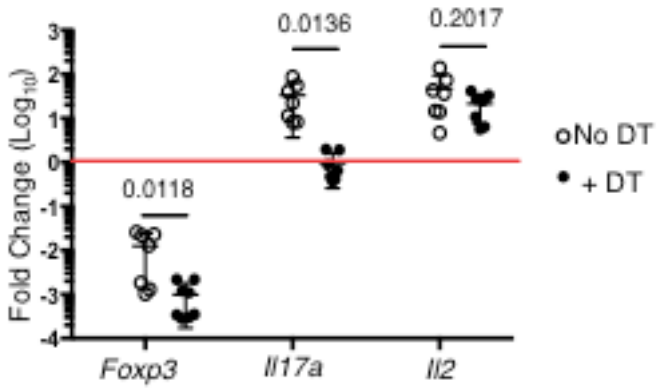
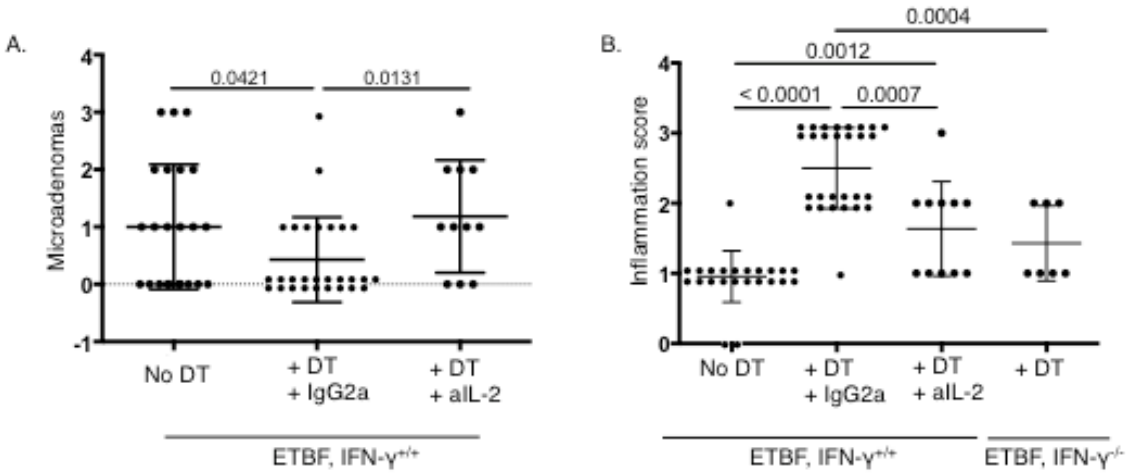


Figure 18: Anti-IL-2 restores ETBF-induced microadenoma formation yet minimizes colitis in the absence of Foxp3^{+} Tregs. B6.Foxp3^{DTR}xMin mice were inoculated with ETBF on day 0. Either 150 µl purified sterile H_2O or 50 ng/g DT was administered ip on days -1, 0, 1, 3, and every other day until sacrifice and harvest. Either rat anti-mouse IL-2 (S4B6-1) or rat IgG2a isotype (JES3-19F) was delivered ip daily (day -1 through day 13). Colons were harvested on day 13, cleaned, rolled, and fixed in 10% formalin for histology & scoring. A) Microadenomas were counted per colon and include 2 separate experiments with 3-12 mice per group per experiment. DT animals treated with isotype Ab (N=10) were not different from DT only-treated animals (N=18), so DT animals treated with or without isotype Ab were combined. Each symbol represents one mouse, and error bars represent 1 standard deviation from the mean in each direction. IFNγ$^{-/-}$ x Min + DT microadenoma counts from **Figure 11** are also shown for easier side-by-side comparison. B) Inflammation scores per colon in A.



CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

Abby L. Geis

September 24, 2015

Educational History:

Ph.D. expected	2015	Program in Immunology	Johns Hopkins School of Medicine
		Mentors: Drew Pardoll, M.D., Ph.D. and Cynthia Sears, M.D.	
B.S.	2005	Biochemistry	Colorado State University

Other Professional Experience

Research Rotation	2008-2009	Lab of Patrizio Caturegli, Johns Hopkins SOM
Research Assistant	2006-2008	Lab of George Eisenbarth, Barbara Davis Center
Research Assistant	2005-2006	Kestrel Labs, Inc.
Research Fellow	2004-2005	Lab of Scott Summers, Colorado State Univ.

Awards and Honors

2015	AAI Trainee Abstract Award, American Association of Immunologists
2014	International Teaching Learning Cooperative Graduate Student Grant, Lilly International Spring Conference On College and University Teaching and Learning
2013	Johns Hopkins Immunology Training Program Annual Retreat Best Poster
2011	Johns Hopkins Immunology Training Program Annual Retreat Best Poster

Publications, peer reviewed

Geis AL, Fan H, Wu X, Wu S, Huso DL, Wolfe JL, Sears CL, Pardoll DM, Housseau F. Regulatory T cell response to enterotoxigenic Bacteroides fragilis colonization triggers IL-17-dependent colon carcinogenesis. Cancer Discovery. 2015; In press.

Landek-Salgado MA, Leporati P, Lupi I, **Geis A**, Caturegli P. Growth hormone and proopiomelanocortin are targeted by autoantibodies in a patient with biopsy-proven IgG4-related hypophysitis. Pituitary. 2012; 15(3):412-9.

Sears CL, **Geis AL**, Housseau F. Bacteroides fragilis subverts mucosal biology: from symbiont to colon carcinogenesis. J Clin Invest. 2014; 124:4166-72.

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Gutenberg A, Landek-Salgado M, Tzou SC, Lupi I, **Geis A**, Kimura H, and Caturegli P. Autoimmune hypophysitis: expanding the differential diagnosis to CTLA-4 blockade. Expert Rev Endocrinol Metab. 2009; 4(6):681-698.

Posters, Abstracts, etc.

Geis AL, Fan H, Wu X, Wu S, Huso DL, Sears CL, Pardoll DM, Housseau F. "A [T] regulatory paradox: Priming an oncogenic inflammatory response to gut microbiota." 2015. Johns Hopkins Center for Autoimmune Disease Research: 17th Annual Autoimmunity Day, Baltimore, MD

Geis AL, Fan H, Wu X, Wu S, Huso DL, Sears CL, Pardoll DM, Housseau F. "Enterotoxigenic Bacteroides fragilis induces oncogenic regulatory T cells." 2015. American Association of Immunologists Annual Meeting, New Orleans, LA

Geis AL, Fan H, Wu X, Wu S, Huso DL, Sears CL, Pardoll DM, Housseau F. "A pro-inflammatory response of mucosal Tregs to enterotoxigenic Bacteroides fragilis promotes IL-17-dependent colon neoplasia." 2015. Keystone Symposium: T cells: Regulation and Effector Function, Snowbird, UT

Geis AL, Fan H, Wu X, Wu S, Sears CL, Pardoll DM, Housseau F. "Stat3 and Th17-mediated colitis promotes regional tumorigenesis in response to a common commensal bacterium: role of Foxp3+ Treg/Th17 balance." 2010. American Association of Immunologists Annual Meeting, Baltimore, MD

Geis AL, Fan H, Wu X, Wu S, Sears CL, Pardoll DM, Housseau F. "A human commensal promotes colorectal cancer in Min mice: role of the Treg / Th17 balance in the colitis-induced carcinogenesis." 2010. Keystone Symposium: Role of Inflammation in Oncogenesis, Keystone, CO

Service and leadership

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2014-2015	Immunology Instructor	Johns Hopkins University
2014	Course Development	Johns Hopkins School of Nursing
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